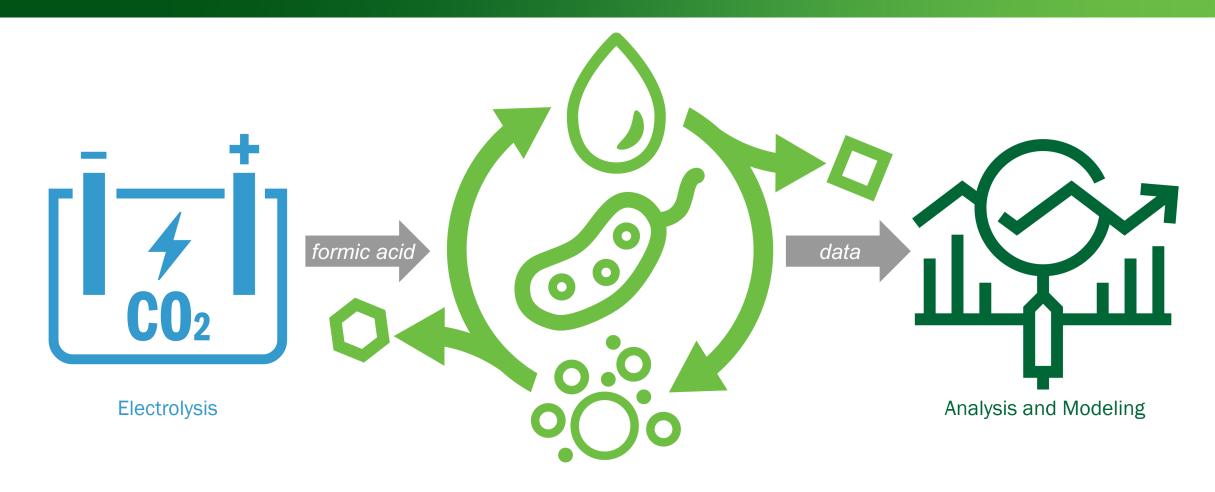


DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review 2.3.2.121 Biological conversion of formic acid for CO2-to-Fuels

April 6, 2023
Carbon Dioxide Utilization
Christopher W. Johnson
National Renewable Energy Laboratory

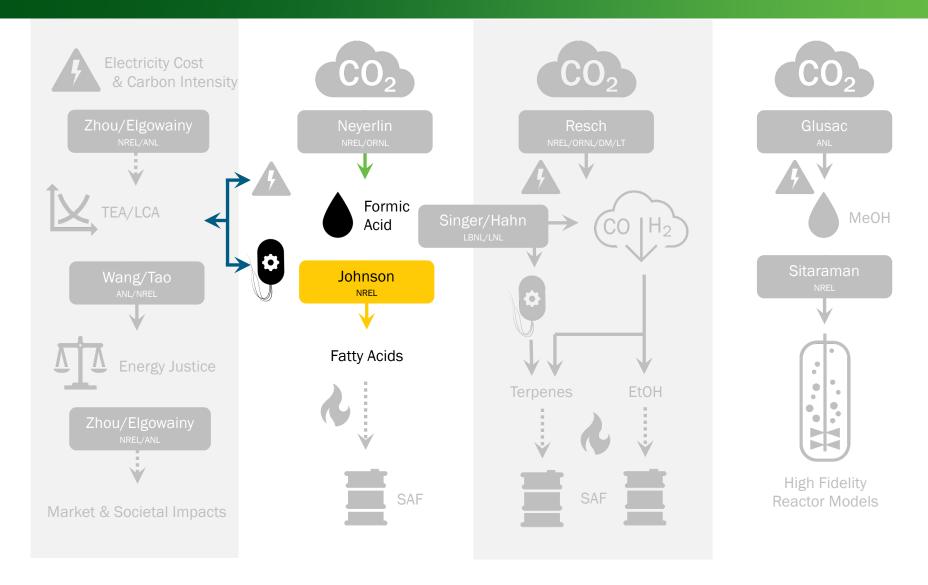
CO₂RUe Consortium



Biological Upgrading

This project builds upon our previous BETO-funded project, 2.3.2.111 Improving formate upgrading by *Cupriavidus necator*

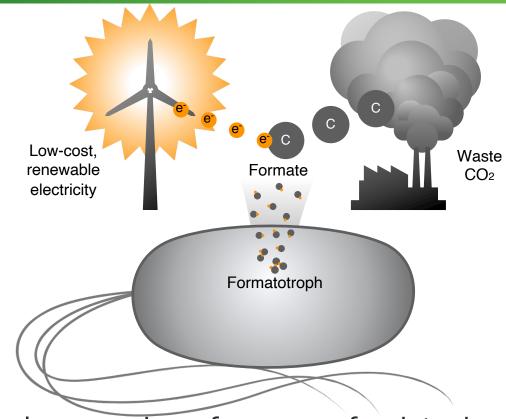
CO₂RUe Consortium





Formic acid as an intermediate for conversion

- Formate/formic acid can be generated by electrocatalytic reduction of CO₂
- Using renewable energy could enable low cost, low GHG production of formate
- Formate can be used as a source of carbon and energy for some bacteria, termed formatotrophs



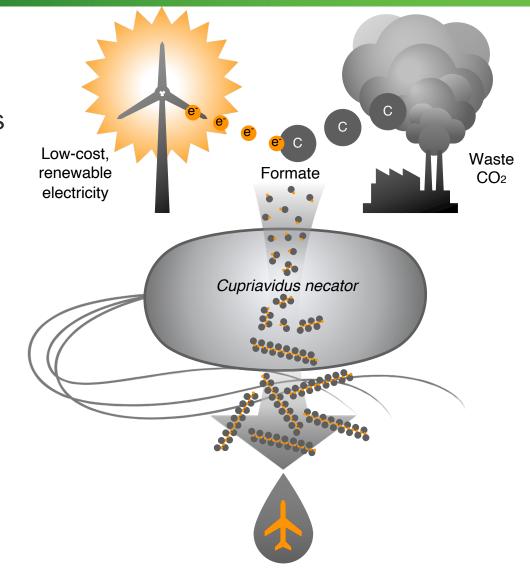
- Overcomes challenges associated with biological conversion of gaseous feedstocks such as solubility, mass transfer, safety, storage, transport, etc.
- Formic acid is preferred to formate as it reduces the accumulation of the cation

Biological conversion of formic acid for CO2-to-Fuels

Project Goal: Develop the soil bacterium *Cupriavidus necator* as a robust microbial host for conversion of formate to fatty acids, which can be converted to sustainable aviation fuel (SAF) and diesel blendstocks

Objectives:

- Develop a process for high density cultivation of C. necator using pH-stat feeding of formic acid
- Perform adaptive laboratory evolution to improved growth of *C. necator* on formic acid
- Engineer C. necator for production of C14 C18 fatty acids from formic acid
- Coordinate with other consortium projects toward integration and impact

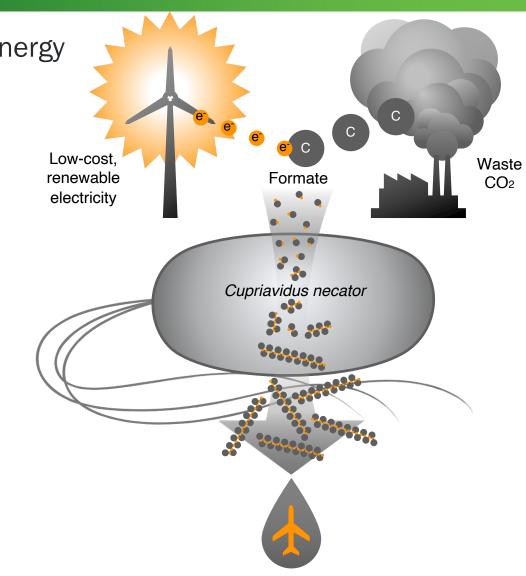




Cupriavidus necator as a host for formic acid conversion

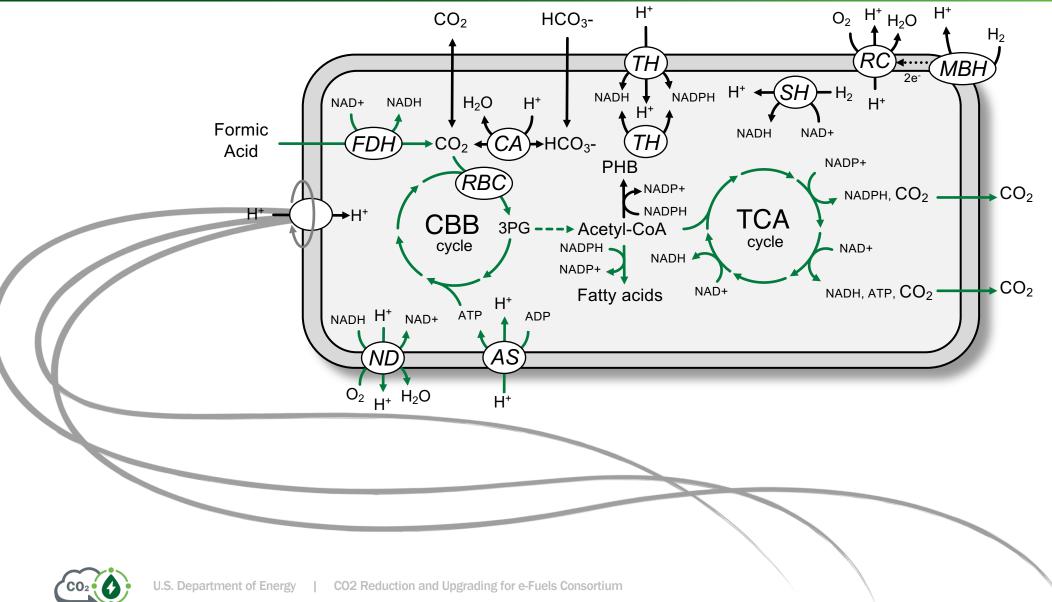
 Aerobic metabolism generates large amounts of energy to make C-C bonds without biproducts (e.g. EtOH, acetate)

- Metabolically versatile
 - Sugars, organic acids, aromatics, etc.
 - CO₂ (for carbon) + H₂ (for energy)
 - Formate (for carbon and energy)
- Grows to high cell densities
 - 281 g/L on glucose (Ryu et al., 1997)
 - 91 g/L on CO_2 + H_2 (Tanaka et al., 1995)
- Facile genome engineering
- Well studied as a model for PHA production
- Proven industrial host for PHA production

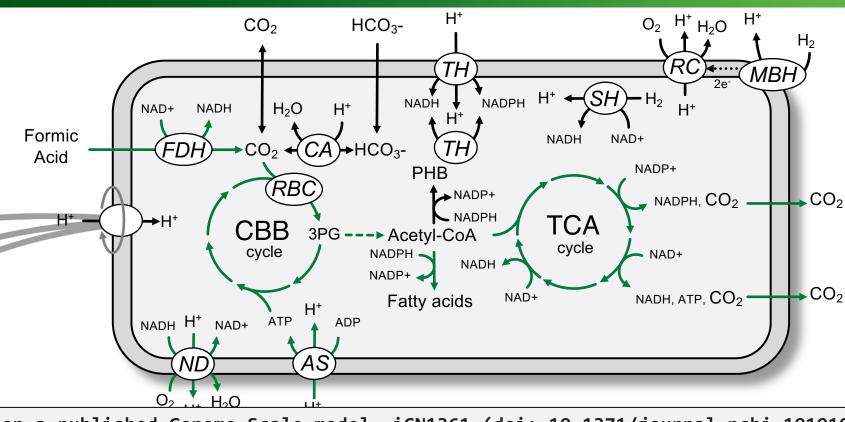




CO₂ and energy metabolism in *C. necator*



CO₂ and energy metabolism in *C. necator*



Based on a published Genome Scale model, iCN1361 (doi: 10.1371/journal.pcbi.1010106):

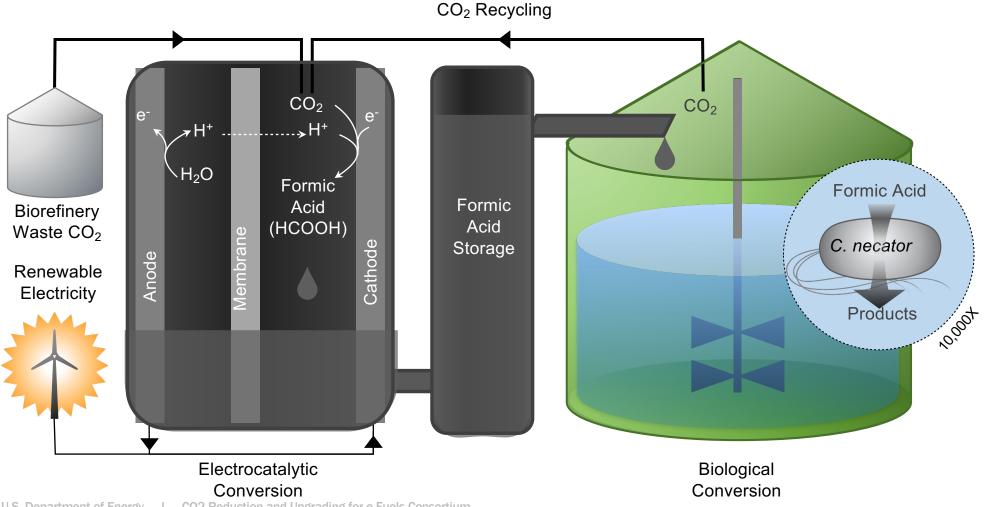
100 formate + 26.57 02 + 5.77 NH4 + 94.93 H+ + 0.4893 HP04 + 0. 089 S02 + 0.002 Fe -> 77.23 CO2 + 92.01 H20 + 0.55014 Biomass

Maximum theoretical yield of biomass from formic acid: 22.73% (Cmol/Cmol)

100 formate + 20.59 02 + 98.72 H+ -> 79.54 CO2 + 79.54 H2O + 1.279 C16 Maximum theoretical yield of C16 fatty acid from formic acid: 20.46% (Cmol/Cmol)

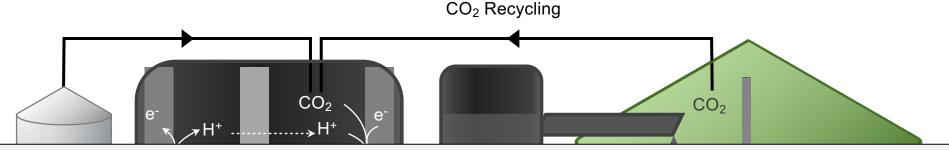
An electro/biocatalytic system for CO₂ conversion

We envision a system in which renewable electricity is used for electrocatalytic reduction of CO₂ to generate a concentrated solution of formic acid for biological conversion.



An electro/biocatalytic system for CO₂ conversion

We envision a system in which renewable electricity is used for electrocatalytic reduction of CO₂ to generate a concentrated solution of formic acid for biological conversion.



Based on a published Genome Scale model, iCN1361, if CO2 was recycled or H2 was supplemented:

100 formate (+ 333.3 H2) + 113.8 O2 + 25.32 NH4 + 77.76 H+ + 2.149 HPO4 + 0. 3907 SO2 + 0.0118 Fe -

398.2 H20 + 2.4159 Biomass

Maximum theoretical yield of biomass from formic acid: 100% (Cmol/Cmol)

100 Formate (+ 392.1 H2) + 97.29 O2 + 93.75 H+ -> 382.1 H20 + 6.25 C16 FA

Maximum theoretical yield of C16 fatty acid from formic acid: 100% (Cmol/Cmol)

In this case, 100% of CO2 would be converted to biomass or C16 FA so the FA/biomass ratio (Cmol/Cmol) is an important metric for this project.

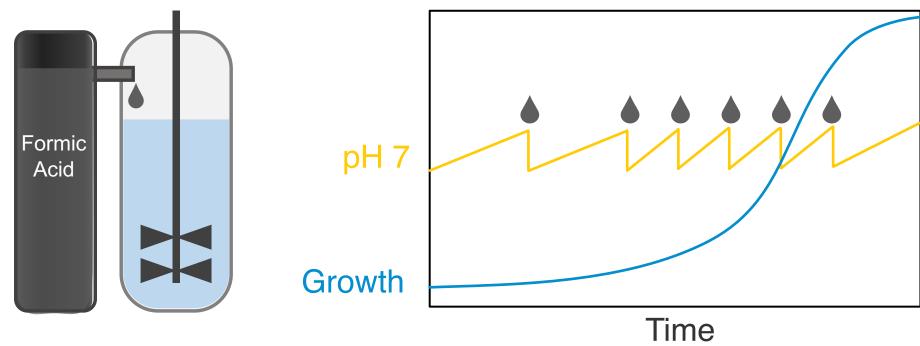


>

1. Approach: Technical

Bioprocess Development

- Optimize cultivation of *C. necator* in laboratory-scale bioreactors with pH-stat feeding of formic acid to establish parameters such as inoculation, feeding rate, and aeration
 - pH maintained by addition of 35% formic acid
 - Formic acid is fed as quickly as it is consumed
 - Concentration of formic acid in the bioreactor is minimized





1. Approach: Technical

Strain Development

- Engineer C. necator to produce fatty acids using well established genome modification methods
 - Deleted a competing pathway for PHB
 - Over expressed an acyl-CoA thioesterase ('TesA) to promote free fatty acid formation
 - Reducing fatty acid degradation by deleting acyl-CoA synthetases
- Evolve C. necator to improve assimilation of, and subsequently conversion of, formic acid
 - Building on previous evolution on formate
 - Grow continuously in a chemostat using pH-stat feeding of formic acid
 - Causative mutations will be identified by full genome resequencing and used to rationally engineer strains with improved growth on formic-acid

Mal-ACP **Fatty Acid Synthesis** acyl-ACP NAD(P)+ Lipids Oils Thioesterase NAD(P)H 'TesA Free Fatty Acyl-CoA **Synthetase β-Oxidation** acyl-CoA **GROWTH**

Modified from Agnew, D.E., Pfleger, B.F., 2013. Synthetic biology strategies for synthesizing polyhydroxyalkanoates from unrelated carbon sources. Chem. Eng. Sci. 103, 58-67.

CO2 Reduction and Upgrading for e-Fuels Consortium

1. Approach: Risks and mitigation

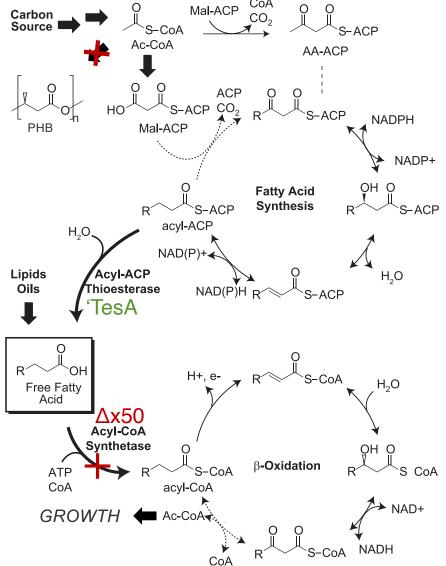
Risk: Fatty acid degradation must be attenuated and is extremely robust in *C. necator* so up to 50 acyl-CoA synthetases might need to be deleted

Mitigation:

- Our current method for knockouts is robust, with a knockout taking about 2.5 weeks
- We can use advanced tools to identify and critical knockouts

Risk: Components/contaminants (e.g. catalyst shedding) in the formic acid stream may affect growth of *C. necator* **Mitigation:**

- The stream could be preprocessed to remove contaminants prior to biological conversion
- C. necator is particularly resistant to metals
 - This could be enhanced using laboratory evolution



Modified from Agnew, D.E., Pfleger, B.F., 2013. Synthetic biology strategies for synthesizing polyhydroxyalkanoates from unrelated carbon sources. Chem. Eng. Sci. 103, 58–67.

1. Approach: Management

Management



Christopher Johnson



Michelle Reed Project Mgmt

Strain Development



Christopher
Calvey
Scientist



Lucas Friedberg Technician



Emm Fulk Postdoc

Coordination

- Lab meetings weekly
- Postdoc one-on-ones biweekly
- CO₂ Biology Working Group (including advisory board members) bimonthly
- BETO / PI meeting bimonthly

Bioprocess Development



Violeta
Sànchez i Nogué
Scientist



Aleena
White
Technician

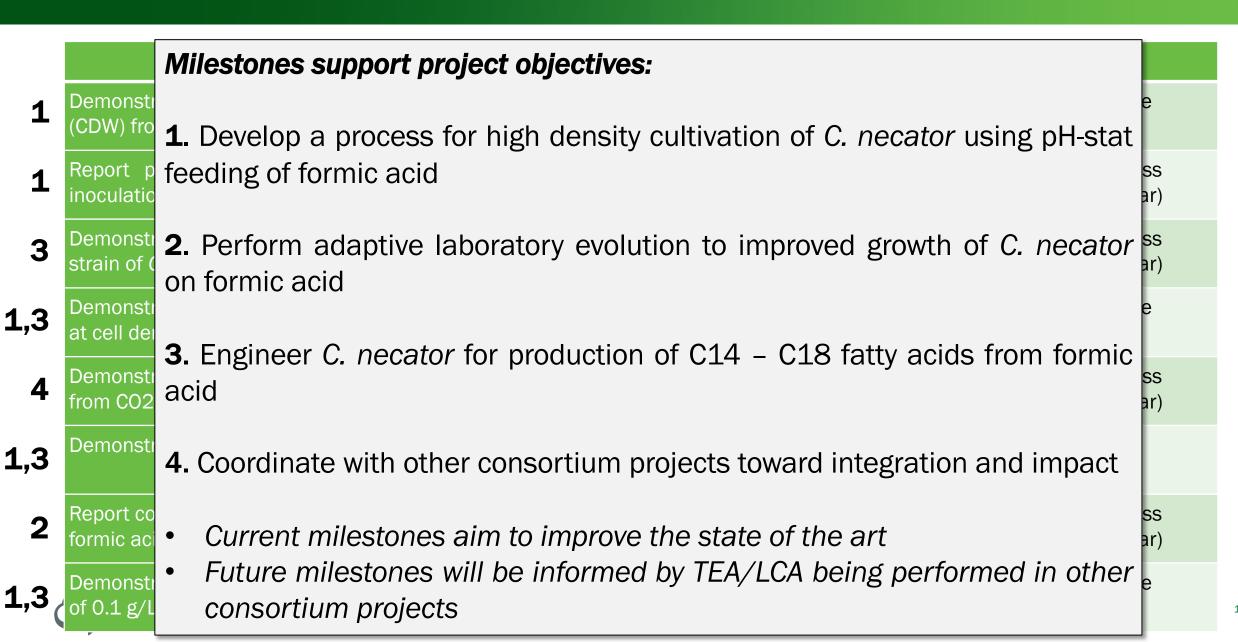


Reuben Swart Postdoc

1. Approach: Milestones

Milestone Name/Description	End Date	Туре
Demonstrated growth of <i>C. necator</i> at cell densities of at least 8 g/L cell dry weight (CDW) from formic acid	3/31/2022	Annual Milestone (Regular)
Report process optimization parameters for pH-stat cultivation including seed inoculation conditions, initial formic acid concentration, and feeding rates	6/30/2022	Quarterly Progress Measure (Regular)
Demonstrated production of 0.01 g/L C14 – C18 fatty acids using an engineered strain of $\it C.\ necator$ in small scale (< 100 mL) shake flasks	9/30/2022	Quarterly Progress Measure (Regular)
Demonstrated growth of a <i>C. necator</i> strain engineered for production of fatty acids at cell densities of at least 8 g/L CDW from formic acid	12/31/2022	Annual Milestone (Regular)
Demonstrate conversion of electrochemically-derived formate/formic acid generated from CO2 by K.C. Neyerlin's project to a \geq C6 product	3/31/2023	Quarterly Progress Measure (Regular)
Demonstrated production of 0.1 g/L of C14 – C18 fatty acids from formic acid.	6/30/2023	Go/No-Go
Report conditions for adaptive laboratory evolution to improve growth of <i>C. necator</i> on formic acid using fully-controlled bioreactors	9/30/2023	Quarterly Progress Measure (Regular)
Demonstrated production of 1 g/L of C14 – C18 fatty acids from formic acid at a rate of 0.1 g/L/h	9/30/2024	Annual Milestone (Regular)

1. Approach: Milestones



2. Progress and Outcomes: Completed Milestones

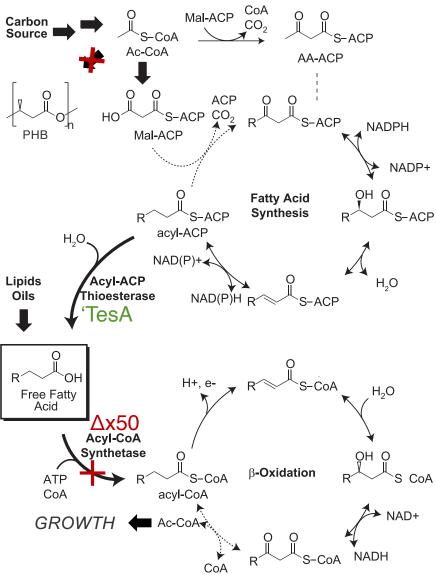
	Milestone Name/Description	End Date	Туре
√	Demonstrated growth of <i>C. necator</i> at cell densities of at least 8 g/L cell dry weight (CDW) from formic acid	3/31/2022	Annual Milestone
√	Report process optimization parameters for pH-stat cultivation including seed inoculation conditions, initial formic acid concentration, and feeding rates	6/30/2022	Quarterly Progress Measure
√	Demonstrated production of 0.01 g/L C14 – C18 fatty acids using an engineered strain of <i>C. necator</i> in small scale (< 100 mL) shake flasks	9/30/2022	Quarterly Progress Measure
√	Demonstrated growth of a <i>C. necator</i> strain engineered for production of fatty acids at cell densities of at least 8 g/L CDW from formic acid	12/31/2022	Annual Milestone
√	Demonstrate conversion of electrochemically-derived formate/formic acid generated from CO2 by K.C. Neyerlin's project to a ≥ C6 product using <i>C. necator</i>	3/31/2023	Quarterly Progress Measure



2. Progress and Outcomes: Fatty acid production

- Genome modification is performed using homologous recombination-based methods that are well established in our lab
- To promote fatty acid production, a competing pathway for polyhydroxybutyrate (PHB) production was deleted, and a thioesterase ('TesA) gene was incorporated
- Engineering of *C. necator* for production of C14 C18 fatty acids has been demonstrated but is limited by their robust ability to degrade them.
- A major focus of our engineering is to delete up to 50 genes encoding acyl-CoA synthetases, so that fatty acids will accumulate rather than being degraded.
- To date we have deleted 8 acyl-CoA synthetase genes.

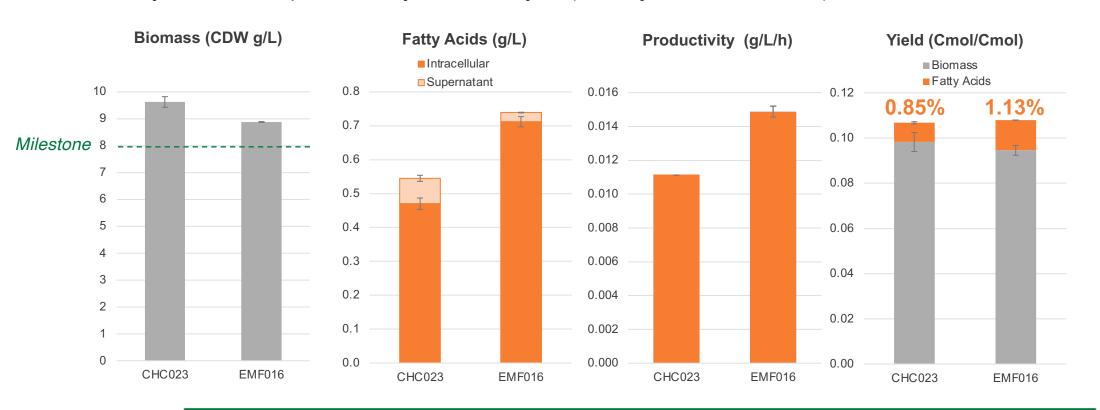




Modified from Agnew, D.E., Pfleger, B.F., 2013. Synthetic biology strategies for synthesizing polyhydroxyalkanoates from unrelated carbon sources. Chem. Eng. Sci. 103, 58–67.

2. Progress and Outcomes: Fatty acid production

- *C. necator* CHC023 was engineered for fatty acid production using a heterologous 'TesA thioesterase and two acyl-CoA synthetase knockouts (EMF016)
- Strains were cultivated in duplicate in 500 mL bioreactors fed 35% formic acid by pH-stat for about 48 hours
- Fatty acids were quantified by FAME analysis (Van Wychen et al., 2015)



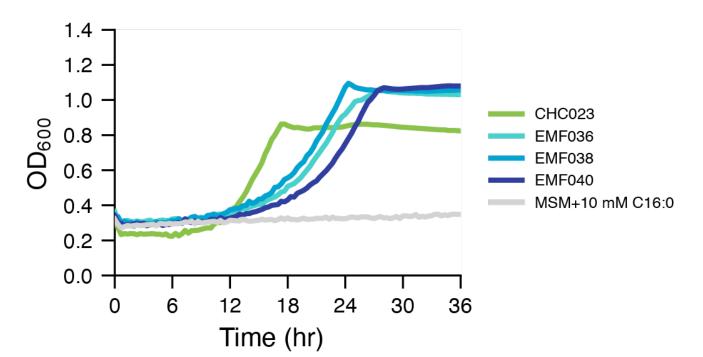


20.46%
(C_{mol}/C_{mol})
Maximum
theoretical yield
assuming no
CO₂ recycling or
supplemental H₂



2. Progress and Outcomes: Fatty acid production

- C. necator engineered with several acyl-CoA synthetase knockouts
- Strains with 5, 6, or 7 acyl-CoA synthetase deletions ($\Delta x5 \Delta x7$) were assayed for their ability to degrade fatty acids by growing them on 10 mM C16 fatty acids as their sole source of carbon and energy in 96 well microtiter plates

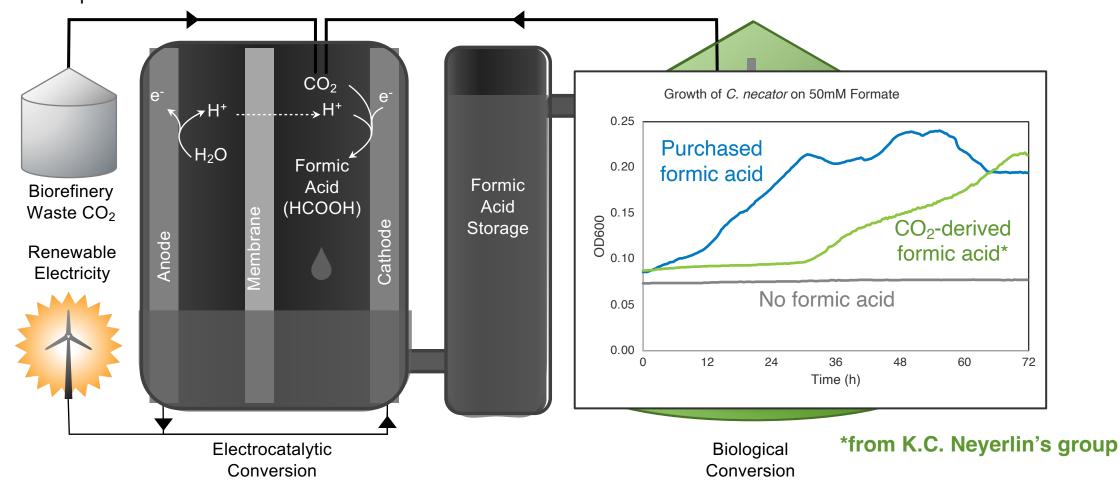


Strain	Max growth rate (OD/min)
CHC023 (parent)	1.76×10 ⁻³ (±2.4×10 ⁻⁴)
EMF036 (Δx5)	1.65×10 ⁻³ (±9.8×10 ⁻⁵)
EMF038 (Δx6)	1.61×10 ⁻³ (±5.6×10 ⁻⁵)
EMF040 (Δx7)	1.62×10 ⁻³ (±7.3×10 ⁻⁵)
	Avg. (± 1 s.d.); n=3



2. Progress and Outcomes: CO₂-derived formic acid

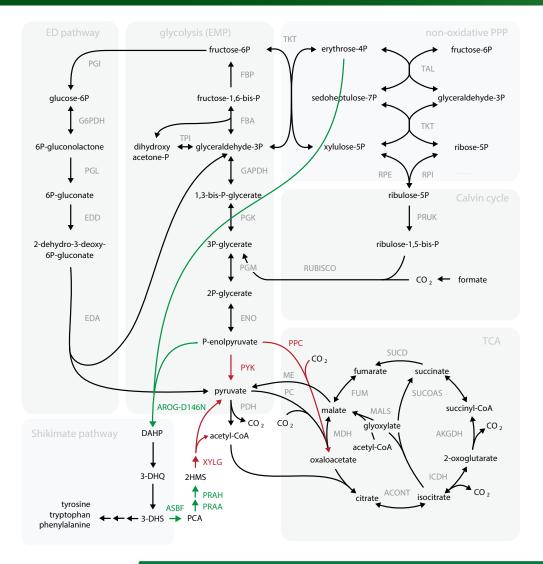
C. necator was grown in 50 mM formic acid generated from CO₂ by K.C Neyerlin's group in a microtiter plate



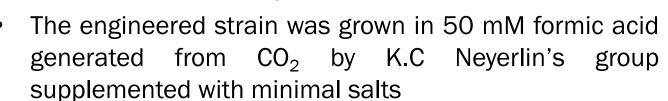


Outcome: C. necator can grow on raw CO₂-derived formic acid

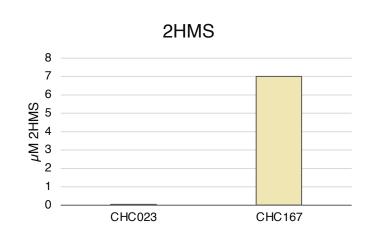
2. Progress and Outcomes: CO₂-derived formic acid

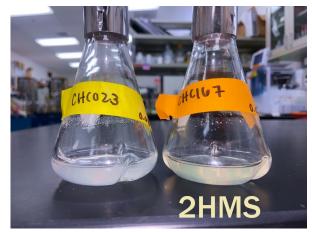


- *C. necator* was engineered to produce the polymer precursor 2-hydroxymuconate semialdehyde (**2HMS**)
 - Introduced production pathway
 - Deleted competing pathways



2HMS was measured by absorbance at 375 nm







2. Progress and Outcomes: Upcoming Milestones

	Milestone Name/Description	End Date	Туре
√	Demonstrated production of 0.1 g/L of C14 – C18 fatty acids from formic acid.	6/30/2023	Go/No-Go
	Report conditions for adaptive laboratory evolution to improve growth of <i>C. necator</i> on formic acid using fully-controlled bioreactors	9/30/2023	Quarterly Progress Measure
	Demonstrated production of 1 g/L of C14 – C18 fatty acids from formic acid at a rate of 0.1 g/L/h	9/30/2024	Annual Milestone



3. Impact

Industrial

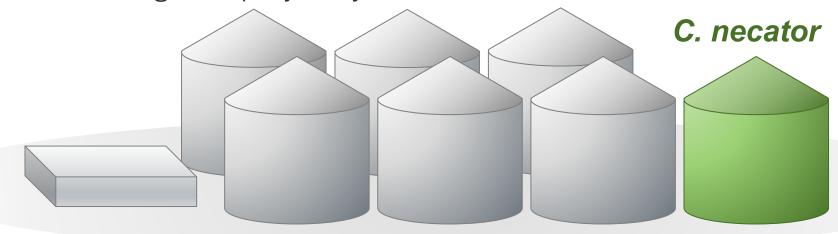
- Generating economic incentive to reduce CO₂ emissions by upgrading it instead
- Developing strains that can improve industrial processes using *C. necator* for production of PHAs as well as food/feed (protein, fats)
 - We have active collaborations with two of companies using C. necator and have engaged with several others
- Anticipate at least one patent application resulting from this work
 - Patent from previously funded project: Metabolic Engineering of *Cupriavidus necator* for improved formate utilization, United States Patent Application No. 18/053696

Scientific

- Contributing to our understanding of autotrophy and formatotrophy and how to improve them
- Invited to speak at the 2021 AIChE Annual Meeting in 2021 and the ACS Annual Meeting in 2021
- Anticipate at least two high-impact peer-reviewed publications resulting from this work
 - Publication from previously funded project: Calvey, C.H., et al., 2023. Improving growth of Cupriavidus necator H16 on formate using adaptive laboratory evolution-informed engineering.
 Metab Eng 75, 78–90. (Impact factor: 8.829)

3. Impact: Biorefinery decarbonization and fuel production

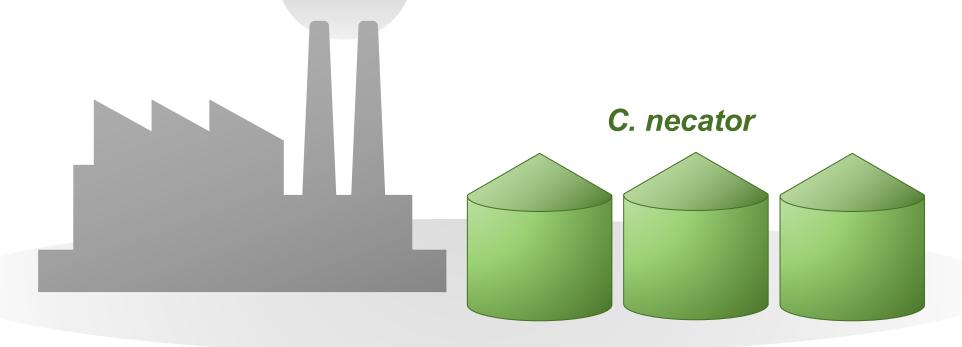
- A typical 40 million gallon/yr starch EtOH biorefinery generates around 15,000 kg CO₂/h
- 1,775,205 L of C. necator would be needed to assimilate CO₂ at the same rate*
- At a 20% yield, this biorefinery would produce C16 fatty acids at 1,092 kg/h (0.642 g/L/h or 9,570 metric tons/yr)
- If this were applied to every ethanol biorefinery in the United States, it could **reduce CO₂ emissions by 45 million tons/yr** and produce **3.2 million tons/yr of fatty acids** that could be subsequently converted to about **1 Billion gallons/yr biodiesel or sustainable aviation fuel (SAF) blendstock**
- BETO's SAF Grand Challenge aims to enable production of 3 billion gallons of SAF per year by 2030 and 35 billion gallons per year by 2050



^{*} Conditions and assumptions: 100% of CO₂ is instantly converted to formic acid. *C. necator* consumes formic acid at a rate of 12 mmol/g cells/h and 16% is assimilated (Grunwald, et al., 2014. doi:10.1111/1751-7915.12149). *C. necator* culture is 100 g/L cells. Fermenters are 2 million L.

3. Impact: Industrial decarbonization and fuel production

- In 2019, the average power plant in the U.S. emitted 39,205 kg of CO₂/h.
- 4,639,794 L of C. necator would be required to assimilate formate at the same rate*
- If this system were applied to every power plant in the US, at a 20% yield it could reduce emissions by 1.7 metric gigatons and generate enough fatty acids to produce about 39.24 billion gallons of fuel per year



^{*} Conditions and assumptions: 100% of CO₂ is instantly converted to formic acid. *C. necator* consumes formic acid at a rate of 12 mmol/g cells/h and 16% is assimilated (Grunwald, et al., 2014. doi:10.1111/1751-7915.12149). *C. necator* culture is 100 g/L cells. Fermenters are 2 million L.

Summary

- Developing the natural formatotroph Cupriavidus necator as a robust microbial host for conversion of formic acid to fatty acids, which can be converted to sustainable aviation fuel and diesel blendstocks
- The maximum theoretical yield of fatty acids from formic acid is 21.5% (Cmol/Cmol) and CO₂ recycling or H₂ supplementation would increase it to 100%
- We have deleted 8 of up to 50 acyl-CoA ligases to prevent degradation of fatty acids and promote their accumulation
- Our *C. necator* engineered for fatty acid production contained heterologous 'TesA thioesterase and two acyl-CoA ligase knockouts, EMF016, produced **0.73 g/L** total lipids from formic acid at **0.015 g/L/h** and a yield of **1.3% (Cmol/Cmol)**
- If applied to every biorefinery in the US at a 20% yield, this system could generate fatty acids for production of up to 1 billion gallons/yr of SAF



Quad Chart Overview: 2.3.2.121

Timeline

Project start date: 1/1/2022Project end date: 9/30/2024

	FY22 Costed	Total Award
DOE Funding	\$412,252	\$3,000,000 (FY22-24)
Project Cost Share*	N/A	N/A

TRL at Project Start: 3
TRL at Project End: 4

Project Goal

Engineer *C. necator* for conversion of formic acid to fatty acids and develop a bioprocess to optimize conversion of formic acid.

End of Project Milestone

Demonstrated production of 1 g/L of C14 – C18 fatty acids from formic acid at a rate of 0.1 g/L/h.

Funding Mechanism

Bioenergy Technologies Office FY21 CO2-To-Fuels Lab Call (DE-LC-000006) – 2021

Project Partners*

CORE Consortium (NREL, ANL, LBNL)

Team Members

- Michelle Reed
- Violeta Sànchez i Nogué
- Christopher Calvey
- Aleena White
- Emm Fulk
- Reuben Swart

BETO Technology Manager

Ian Rowe

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Office of ENERGY EFFICIENCY & RENEWABLE ENERGY

BIOENERGY TECHNOLOGIES OFFICE

Q&A





Additional Slides

Responses to Previous Reviewers' Comments

NOTE: These comments were regarding the previous BETO-funded project, 2.3.2.111 Improving formate upgrading by *Cupriavidus necator*, that this current project builds upon

"The selection of 2HMS product may not be the most appropriate product but is sufficient for this proof-of-principle project" and "The choice of 2-HMS as a target was not adequately justified. Given that there is no existing market for 2-HMS..."

• While we agree that 2-hydroxymuconate semialdehyde (2HMS) was a useful target as a proof-of-principal due to it ease of detection (yellow color) in the current project we have pivoted to targeting production of fatty acids, which are in better alignment with BETO goals as they can be readily converted to fuels using established methods.

"While the study was thorough and insightful, it lacks the investigation of potential complexities of such biological systems."

• While such complexities were not addressed in our BETO Peer Review 2021 presentation, we have since analyzed the results of transcriptomic experiments to understand the mechanisms by which formate conversion was improved in strains evolved as part of our previous project. Based on this data, we were able to provide a mechanistic understanding of mutations that arose during evolution and recently reported these findings in an article published in *Metabolic Engineering*.

Publications, Patents, Presentations, Awards, and Commercialization

Publications

• Calvey, C.H., et al., 2023. Improving growth of *Cupriavidus necator* H16 on formate using adaptive laboratory evolution-informed engineering. Metab Eng 75, 78–90. (Primarily from previous BETO-funded formate conversion project this current project builds upon)

Patent Applications

 Metabolic Engineering of Cupriavidus necator for improved formate utilization, United States Patent Application No. 18/053696 (Primarily from previous BETO-funded formate conversion project this current project builds upon)

Presentations

- 2021 American Institute of Chemical Engineers (AIChE) Annual Meeting (From previous BETO-funded formate conversion project this current project builds upon)
- 2023 American Chemical Society (ACS) Annual Meeting

